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Effect of diet of Varying Protein concentrations on the Activity of Erythrocyte Membrane $Ca^{2+}Mg^{2+}$ ATPase in Dogs

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Summary: Alterations in protein diet have been reported to result in alterations in calcium homeostasis in the body. $Ca^{2+}Mg^{2+}$ATPase is an ubiquitous enzyme important in calcium homeostasis in the body. The effect of varying protein diet on the activities of Ca^{2+} pump across cell membranes is however yet to be fully elucidated. In this study, the activity of erythrocyte membrane calcium pump in response to varying protein concentration in diet was therefore studied in the dog. The study was carried out in 24 dogs, randomly divided into 4 groups. The groups were fed with diets containing 30%, 26%, 16% and 0% proteins (high, medium, low and zero) for six weeks respectively. Blood samples were collected from each animal to determine packed cell volumes, hematocrit, blood urea, electrolyte studies and erythrocyte ghost membrane studies. The effects of Ca^{2+} and ATP on the activity of $Ca^{2+}Mg^{2+}$ ATPase were determined in the isolated ghost membrane. The result of the study shows that there was a protein diet dependent increase in the activity of $Ca^{2+}Mg^{2+}$ ATPase in the presence and absence of ATP in all the groups with the highest activity recorded in the high protein diet group and the lowest activity observed in the zero protein group. There was also a protein diet dependent increase in the protein concentration of the membranes in all groups observed with the highest protein concentration recorded in the high protein diet group and the lowest activity observed in the zero protein group.. There was a significant decrease in K^+ concentration ($P<0.05$) and a significant increase in urea concentration of animals fed with high protein diet ($P<0.05$). There was also a significant increase ($P<0.05$) in HCO_3^- concentration in the animals fed with medium protein diet and no significant difference in the PCV and heamatocrit values in all groups. This study has shown that high protein diets increase the activity of the $Ca^{2+}Mg^{2+}$ ATPase in the presence and absence of ATP.

Keywords; *Protein diet, Membrane proteins, $Ca^{2+}Mg^{2+}$ ATPase activity, Calcium.*
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INTRODUCTION

Alterations in dietary conditions have been reported to result in a wide variety of physiological and pathological changes in the body system (Bidlack, 1996). Alterations in diet have also been reported to result in changes in the internal milieu and the extracellular fluid, which surround individual cells. Alterations in the extracellular fluid often lead to changes in concentration, composition, and configuration of membrane transporters.

Proteins form an essential component of a balanced diet because they are an important source of amino acids for the body. These amino acids are reported to be important in a variety of biochemical pathways (Brosnan, 2003)). Alterations in protein diet consumed by individual have been reported to lead to changes in calcium metabolism (Johnson *et al* 1970; Younghee and Hellen 1979; Kerstetter *et al*, 2003). Protein rich diets have also been reported to lead to increased absorption of calcium from the gut, hypercalciuria and subsequent loss of calcium in urine (Kerstetter *et al*, 2003). Diets low in protein on the other hand has been reported to result in reduced calcium absorption and hyperparathyroidism (Kerstetter *et al*, 2003; 2003).

Calcium is essential for many important processes in the body which include bone formation, nerve depolarization, signal transduction, muscle contraction and neuronal function (Hoenderop *et al* 2005). Maintenance of electrochemical gradient across the plasma membrane is also calcium dependent (Hoenderop *et al*, 2005). Calcium pumps are therefore important in the sense that they direct the flow of calcium ions through the plasma membrane or organelle membranes and the resulting gradients are used in a variety of signaling systems mediated by gated ion channels (Bautista and Lewis, 2004). Calcium pumps are ATPases that transport

ions across membranes using energy obtained from the hydrolysis of ATP (Hoenderop *et al*, 2005). The effect of varying protein diets on the activities of calcium pumps is however yet to be fully elucidated.

The erythrocyte membrane was used as a model for this membrane studies because:

1. It is readily available and usually in sufficient quantities for analysis.
2. It is simple to prepare pure membranes from whole blood samples because red cells lack organelles and therefore have only the simple plasma membrane round the cytoplasm. Also, almost all the cytoplasmic content of the erythrocytes can be released by osmotic haemolysis to give ghosts which are pure plasma membranes.
3. They can be transformed to inside out vessels and the exposed inner surfaces can easily be studied. Sarkadi *et al* (1978)

MATERIALS AND METHODS

Animals

Twenty four weaned puppies were used for this study. The animals were housed in well aerated cages at the animal house in the Department of Physiology, University of Ibadan. They were randomly divided into 4 groups of 6 animals per group. The groups were fed with compounded diet of varying percentage of crude protein for six weeks as follows:

Group 1 – Animals in this group received high protein (30.1%) diet.
Group 2 – Animals in this group received medium protein (26.5%) diet.
Group 3 – Animals in this group received low protein (16.1%) diet.
Group 4 – Animals in this group received No (0%) protein diet.

Collection of Blood samples

A total of 20mls of blood was collected from the brachial vein of the forelimb of each animal. Of this, 15mls of blood samples was collected into sample bottles filled with acid citrate dextrose buffer in the ratio 4 volumes of blood to one volume of buffer and used for ghost membrane studies, while the remaining 5mls was collected into lithium heparin bottles for hematological analysis and blood electrolyte studies. All blood samples collected were used within 24 hours of collection.

Preparation of Erythrocyte Ghost Membrane

Hemoglobin free, calmodulin depleted red cell membranes were prepared by the procedure of Niggli *et al* (1981) which is based on the principle of hypotonic lysing developed by Dodge *et al* (1963) involving repeated centrifugation at low ionic strength. All stages of the erythrocyte ghost membrane separation were carried out at $4^{o}C$ to preserve the functional and structural integrity of the membrane.

Determination of Standard Phosphate Curve

The inorganic phosphate liberated was estimated using standard phosphate curve (Stewart, 1974) based on coloured reaction developed using 1.25% ammonium molybydate and 9% ascorbic acid.

Protein Concentration of Erythrocyte Ghost Membrane

Erythrocyte ghost membrane protein was estimated by the procedure of Lowry *et al* (1951) using bovine serum albumin (BSA) as standard.

Heamatological Analysis

Improved Neubauers haemocytometer (Dacie and Lewis, 1984) was used for the blood cell count. Hawksley microcapillary tube, microcentrifuge and microheamatocrit reader were used for estimating the packed cell volume. Sahli's apparatus was used for hemoglobin concentration.

Measurement of Plasma Sodium, Potassium, Chloride and Bicarbonate

The remaining blood samples collected were centrifuged at 300 rpm for 5 min. The plasma was aspirated and used for the measurement of plasma electrolyte. Plasma sodium and potassium, were analyzed by flame absorption technique using a flame photometer [SEAC flame Photometer spectrophotometer Model FP20], while plasma bicarbonate ion and chloride ion concentration were assessed using colorimetric method as described by Doubas *et al*, (1971).

Statistical Analysis

Data obtained for each group of experimental rats in the various parameters determined were expressed as the mean ± SEM. Statistical comparisons between the groups were made using the Student T-test. The level of significant difference between the groups was evaluated at $P \leq 0.05$ at each level.

RESULTS:

Protein Concentration of Erythrocyte Ghost Membrane (Table 1): There was a significant increase ($P<0.01$) in the protein concentration of the erythrocyte ghost membrane of animals fed with a

high protein diet when compared with the no protein diet group. In addition to this, there was a 50% and a 75% increase (P<0.05) in the protein concentration of erythrocyte ghost membrane isolated from the low protein group and medium protein group when compared with the no protein group. This implies that increases in the protein concentration of the diet led to a concomitant increase in protein concentration of erythrocyte ghost membrane.

Activity of the $Ca^{2+}Mg^{2+}$ ATPase in the absence and presence of ATP (Table 1): The result showed that there was an increase in the activity of $Ca^{2+}Mg^{2+}$ ATPase in the presence and absence of ATP. The observed increase in activity was found to be diet dependent with the highest activity recorded in the high protein diet group and the lowest activity observed in the no protein group.

Effect of Varying Protein Diet on blood Potassium ion Concentration (fig 1): There was a decrease in the K^+ ion concentration of animals fed with the high protein diet after the duration of experiment when compared to before the experimental procedures occurred. There was however no significant change in the K^+ ion concentration of the other groups.

Effect of Varying Protein Diet on blood Urea Concentration (Table 1), Hemoglobin Concentration, Red Blood Cell Count, Packed Cell Volume (Tables 2), Chloride and Sodium ion Concentration (fig. 3):
There was a significant increase (P<0.05) in the urea concentration of animals fed with high protein diet while there were no significant differences in the urea concentration before and after experimental procedures in other groups.

Table 1:
Protein Concentration of Erythrocyte Ghost Membrane of the $Ca^{2+}Mg^{2+}$ ATPase activity and Urea Concentration in Animals fed High Protein, Medium Protein, Low Protein and Zero Protein diet.

Group	Total protein concentration (mg/dl)	Activity of Ca2+ Mg2+ ATPase (µmole pi/µg)		Urea concentration (mg/100ml)	
		in absence of ATP	in presence of	Before treatment	After treatment
High protein	90±4.5**	2.7±0.5	2.5±0.6	23.0±3.5	35.5±1.5[b]
Medium Protein	70±5.5*	2.5±0.2	2.3±0.7	14.3±1.5	17.7±1.1[a]
Low Protein	60±5.3*	2.2±0.6	2.2±0.6	16.0±2.8	17.5±2.1
No Protein	40±2.3	1.8±0.8	2.1±0.8	13.7±1.1	14.7±2.1

Values are Mean± SEM. Asterisks indicate values that are significantly different from the No protein diet fed animal values (= P<0.05; **=P<0.01). [a, b] indicate values that are significantly different from corresponding values obtained before commencement of experiment ([a] = P<0.05; [b] = P<0.01).*

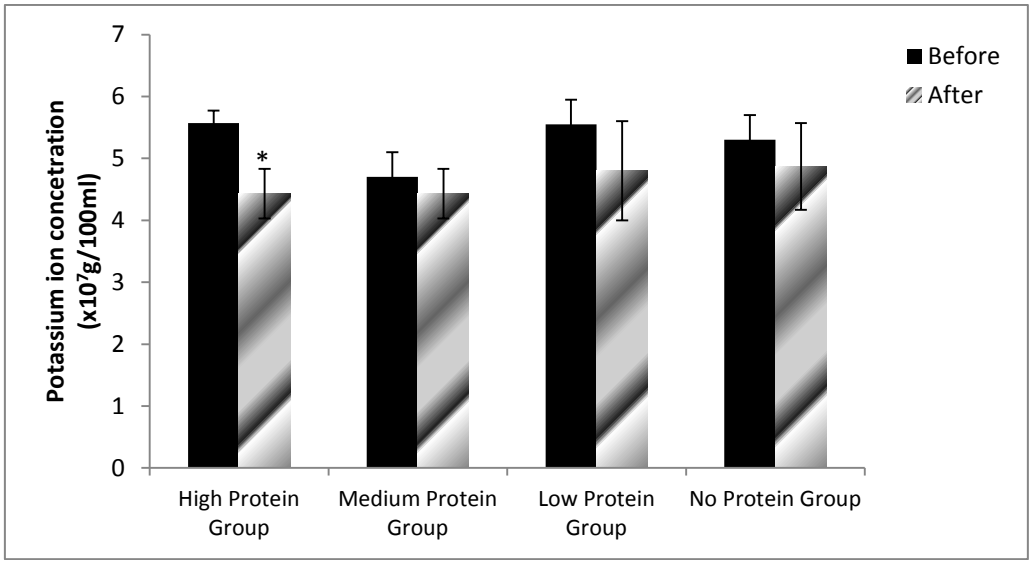

Fig. 1:
Effect of Varying Protein Diet on Potassium ion Concentration. Values are mean±SEM. Asterisks indicate values that are significantly different from corresponding values obtained before commencement of experiment (* = P<0.05).

Table 2:
Blood Hemoglobin Concentration, Red Blood Cell Count and Packed Cell Volume in animals fed varying protein diets

Group	Hemoglobin before (mg / 100ml)	Hemoglobin after (mg / 100ml)	Red Blood Cell Count before (X 10^6mm^3)	Red Blood Cell Count after (X 10^6mm^3)	Packed Cell Volume before (%)	Packed Cell Volume after (%)
High protein	8.60±3.3	9.80±1.0	2.60±1.2	3.10±0.2	32.7±1.9	37.3±3.5
Medium Protein	9.70±1.40	8.90±0.5	2.80±0.5	2.63±0.1	34.3±4.7	34.0±2.0
Low Protein	9.01±1.20	9.07±1.20	3.27±0.1	2.97±0.1	37.7±1.5	35.7±1.1
No Protein	9.30±1.70	9.30±1.70	2.83±0.5	2.90±0.3	32.7±4.9	35.3±6.1

Values are mean ± SEM. There was no significant difference in the values of parameters measured.

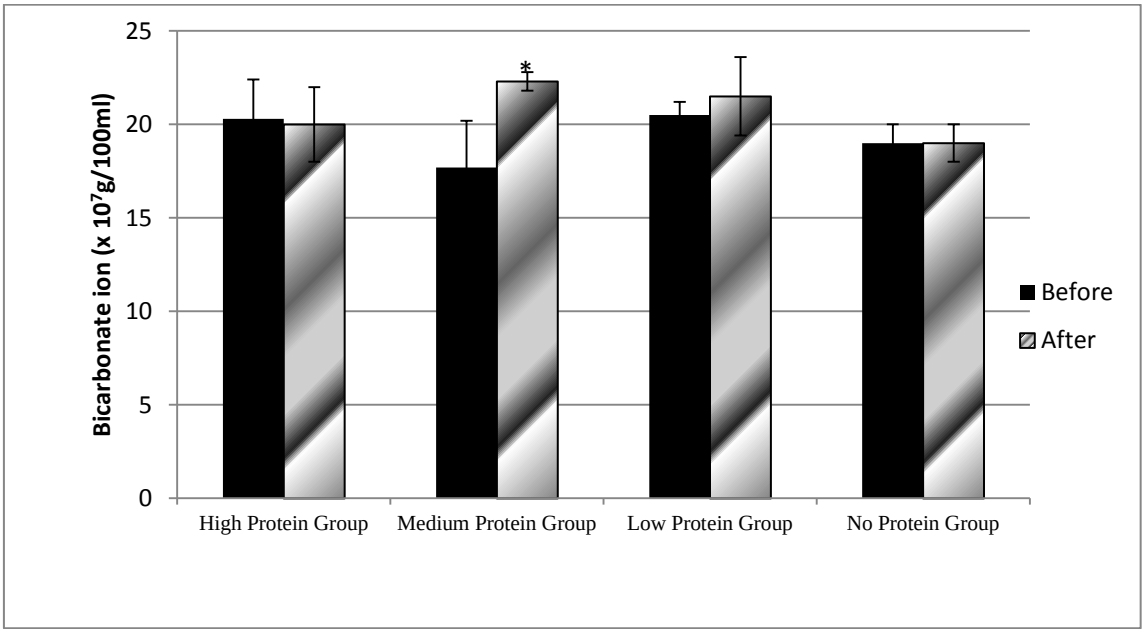

Fig 2:
Effect of varying Protein Diet on Bicarbonate ion Concentration. Values are Mean ± SEM. Asterisks indicate values that are significantly different from corresponding values obtained before commencement of experiment (* = P<0.05).

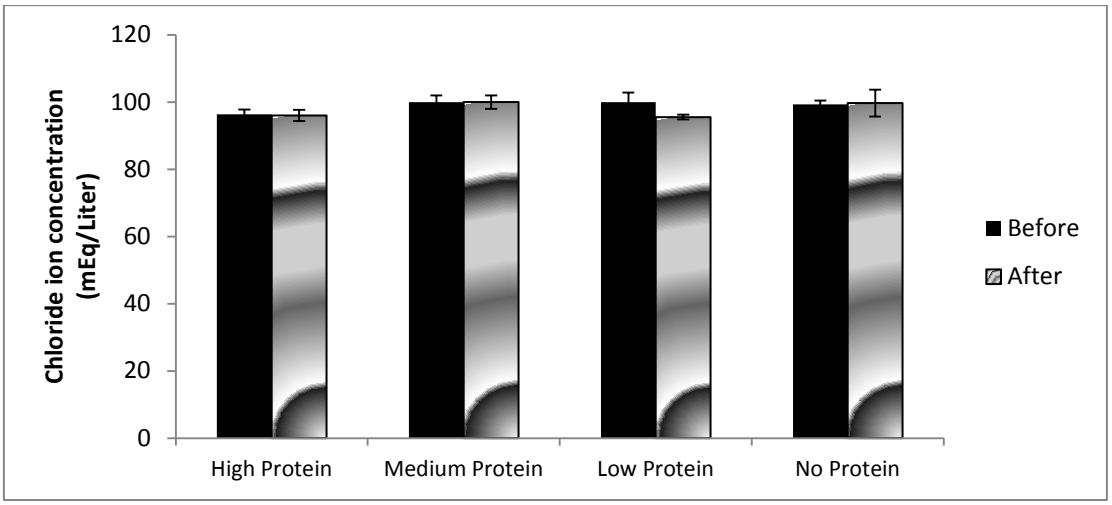

Fig. 3:
Effect of varying Protein Diet on Blood Chloride ion Concentration. Values are Mean ± SEM. There was no significant difference in the values of parameters measured.

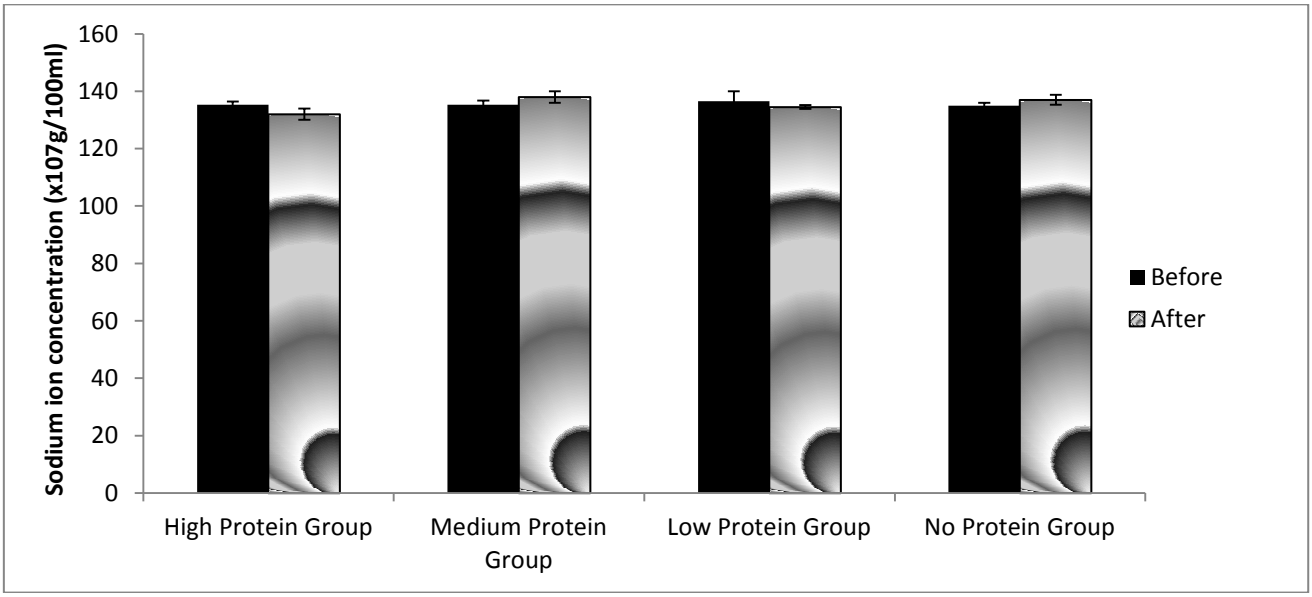

Fig. 4:
Effect of varying Protein Diet on Sodium ion Concentration. Values are Mean ± SEM. There was no significant difference in the values of parameters measured.

Effect of Varying Protein Diet on blood Bicarbonate ion Concentration (fig 2):
There was a significant change in the bicarbonate ion concentration of animals fed with medium protein diet, while the differences in the other groups were not statistically significant.

DISCUSSION

This study has shown that there was an increase in the activity of $Ca^{2+}Mg^{2+}$ ATPase in the presence and absence of ATP. There was also an increase in the protein concentration of the membranes. The observed increase in activity and membrane protein concentration were found to be diet dependent with the highest activity recorded in the high protein diet group and the lowest activity observed in the no protein group. This is in accordance with Olorunsogo *et al*, (1989) who reported differences in protein concentration of membrane of healthy children when compared with children having kwashiorkor.

It has also been reported that protein rich diets may lead to increased absorption of calcium from the gut, hypercalcuria and subsequent loss of calcium in urine (Kerstetter *et al* 2003; 2003). This is because excess protein increases the body's acid load which is buffered or neutralized in part by the bony skeleton, thus releasing calcium into the general circulation (Barzel and Massey, 1998; Kerstetter *et al* 2003; 2003). This extra serum calcium is eventually excreted by the kidneys into the urine, thus causing hypercalcuria. Acid loading also directly inhibits renal calcium reabsorption, resulting in an increase in urinary calcium excretion (Kerstetter *et al* 2003; 2003). Hypercalciuria has been reported to stimulate an increase in the activity of the $Ca^{2+}Mg^{2+}$ ATPase (Bianchi *et al*, 1988; Heguilen *et al*, 2009). The observed increase in the membrane protein concentration and activity of the $Ca^{2+}Mg^{2+}$ ATPase in the presence and absence of ATP may therefore not be unconnected to the hypercalciuria caused by increase in the protein diet.

The increase in blood urea concentration as a result of increase in diet protein concentration observed in the study is in accordance with Eggum (1970) and Bassily *et al* (1982) who reported that the blood urea content increases as the protein content of the diet increases. This is because amino acids from ingested diet are either used to synthesize proteins and other biomolecules or when used as a source of energy, it is oxidized to urea and carbon dioxide.

Within the duration of this study, increases in the protein diet did not appear to have any significant effect on hematological parameters and this is in accordance with the study of Johnson *et al* (2001).

In summary, this study shows that high protein diets may lead to an increase in the activity of the $Ca^{2+}Mg^{2+}$ ATPase in the presence and absence of ATP. It also has also shown that an increase in protein diet may lead to an increase in membrane

protein concentration. This effect may be due to hypercalcaemia which has been reported to occur in high protein diets.

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